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High-resolution structural analysis of biolistic transgene integration into the genome of wheat

Abstract Transformation of plant genomes by biolistic methods has become routine over the past decade. However, relatively little is known about how transgenes are physically integrated into the host genome. Using a high-resolution physical mapping technique, fluorescence in situ hybridization on extended DNA fibers (fiber-FISH), 13 independent transgenic wheat lines were analyzed to determine the structural arrangement of stably inherited transgenes in host-plant chromosomes. Twelve transgenic lines were transformed with a single plasmid and one line was co-transformed with two separate plasmids, which co-segregated genetically. Three basic integration patterns were observed from the fiber-FISH experiments: Type I, large tandemly repeated integration; Type II, large tandem integrations interspersed with unknown DNA; and Type III, small insertions, possibly interspersed with unknown DNA. Metaphase FISH showed that the integration of transgenes was in both hetero- and euchromatic, as well as proximal, interstitial and distal, regions of the chromosomes. In the transgenic plants, the type of promotor used, rather than the chromosomal site of transgene integration, was most critical for transgene expression. The integration of the transgenes was not associated with detectable chromosomal rearrangements.

Keywords Fiber-FISH · Physical mapping · Transgene structure · Wheat

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Introduction

Transformation of plant genomes by biolistic methods or by *Agrobacterium* has become routine in plant research. Almost every species of economic importance is amenable to transformation, and the number of transformed crops being registered for testing and commercial production is increasing rapidly. Transformation of crop genomes is desirable for many purposes: increased nutritive value, plant disease resistance, insect resistance, herbicide resistance, and production traits.

Even though transformation has become fairly routine, little is known about how transgenes physically integrate into host genomes. Both the *Agrobacterium* and biolistic methods of transformation have resulted in the integration of multiple, rearranged copies of the transgene at a single physical locus. Multiple copies of the transgene can be head to head, head to tail, truncated, or interspersed with unknown DNA (Takano et al. 1997; Kohli et al. 1998, 1999; Pawlowski and Somers 1998). The mechanism by which multiple copies of a transgene are integrated into one physical locus by either method of transformation is not well understood. In biolistic transformation, it has been hypothesized to be due to either integration at replication forks, resulting in interspersed copies (Pawlowski and Somers 1998), or the multimerization of transgenes in the plant cell prior to integration into the host genome (Kohli et al. 1998).

Traditionally, the copy number and structural information of stably inherited transgenes are analyzed by Southern blot analysis using restriction enzymes that cut the plasmid once. Alternatively, parts of the transgenes

may be used as primers to determine, at the sequence level, how the transgenes are integrated. Additionally, fluorescence in situ hybridization (FISH) using the transgene as a probe to metaphase chromosomes can be done to determine where the transgene is integrated (Hoopen et al. 1996). However, because of the multimerization of the plasmids and structural rearrangements of both the transgenes and the host DNA, the utility of these techniques is limited. We describe here a method whereby the transgenes are physically mapped directly on genomic DNA fibers isolated from the transgenic plants (fiber-FISH). This method approaches the resolution of the Watson-Crick double helix (approximately 2.94 kb/µm) and yields information as to the physical structure of the transgene locus. This method is complementary to Southern blot analysis, sequence analysis, and FISH of metaphase chromosomes.

In the present study, 13 transgenic lines of common wheat, *Triticum aestivum* L., produced by Chen et al. (1998, 1999) using biolistic bombardment, were analyzed by C-banding, metaphase FISH, and fiber-FISH to determine physically the mode of integration of the transgene into the host genome.

Materials and methods

Seventeen transgenic wheat lines cv 'Bobwhite' were produced by biolistic bombardment (Chen et al. 1998, 1999), 13 of which were used in the following experiments. Twelve lines were transformed with the plasmid pAHG11, encoding a rice, *Oryza sativa* L., chitinase gene (*chi11*) under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter, and the *bar* gene as the selectable marker under the control of the maize, *Zea mays* L., ubiquitin promoter (Fig. 1). Line 14 was co-transformed the with plasmids pAHG11, described above, and pGL2ubi-tlp that encodes a rice thaumatin-like protein (*tlp*) driven by the ubiquitin promoter with a hygromycin phosphotransferase gene (*hpt*) driven by the CaMV 35S promoter as a selectable marker (Fig. 1).

Root-tips of T_2 or T_3 transgenic plants were removed and placed in ice-water for 24 h and then fixed in 3:1 (v:v) ethanol:glacial acetic acid. Squash preparations were made in 45% acetic acid and coverslips were removed after freezing on dry ice. Alternatively, meristematic portions of the root tip were disected onto a microscope slide and squashed in the presence of 45% acetic acid before coverslips were removed after freezing on dry ice. The preparations were then dehydrated for 5 min, or overnight, in 100% ethanol before being used for FISH and C-banding. The C-banding procedure and chromosome identification were according to Gill and Kimber (1974) and Gill et al. (1991).

DNA fibers for fiber-FISH were prepared from wheat nuclei isolated according to previously published protocols (Zhong et al.

1996; Jackson et al. 1998). Fibers were prepared on poly-L-lysine coated slides (Sigma) and were used immediately for FISH.

FISH on metaphase chromosomes was done according to previously published protocols (Heslop-Harrison et al. 1991; Pederson et al. 1997). The whole plasmid pAHG11 was used as a probe and labeled with digoxigenin-11-dUTP or biotin-16-dUTP (Boehringer Mannheim) using nick translation according to the manufacturer's protocols. For chromosome identification and for determining the integration into regions of constitutive heterochromatin, two highly repetitive, genome-specific DNA clones were used for FISH and fiber-FISH. Clone pSc119 contains a 120-bp repeat derived from *Secale cereale* L. (Bedbrook et al. 1980) and clone pAs1 contains a 1-kb repetitive DNA sequence cloned from *Aegilops tauschii* Tausch. (Rayburn and Gill 1986). For FISH and fiber-FISH the hybridization solution contained 1 to 2 ng/µl of probe DNA, 300 ng/µl of sheared salmon sperm DNA, 10% dextran sulfate, 50% formamide, and 2 × SSC; 30 µl of the hybridization mixture was applied to each slide. The probe DNA was allowed to hybridize overnight at 37° C to the target DNA. Post-hybridization washes were done in 2 × SSC at 42° C for 10 min, 50% formamide in 2 × SSC at 42° C for 10 min, and 2 × SSC at 42° C for 10 min.

For FISH to metaphase chromosomes, digoxigenin-labeled plasmids were detected with sheep anti-digoxigenin-FITC (Boehringer Mannheim) and biotin-labeled plasmids with rabbit anti-biotin FITC (Vector Laboratories). Chromosomes were counterstained with 4′, 6′-diamidino-2-phenylindole (DAPI) or PI (propidium iodide) in Vectashield (Vector Laboratories). For fiber-FISH, biotin-labeled plasmids were detected with FITC-avidin (Vector Laboratories), followed by goat biotin-anti-avidin (Vector Laboratories) and finally with FITC-avidin (Vector Laboratories), while digoxigenin-labeled plasmids were detected with mouse anti-digoxigenin antibody (Boehringer Mannheim), followed by digoxigenin-anti-mouse antibody (Boehringer Mannheim) and finally by rhodamine-anti-digoxigenin antibody (Boehringer Mannheim), using previously published protocols (Jackson et al. 1998). Slides were visualized with an epifluorescence Zeiss Axioplan 2 microscope. Digital images were captured using a Spot CCD (charge coupled device) camera operated with Spot 2.1 software (Diagnostic Instruments) and processed with Adobe Photoshop v5.5 (Adobe Systems Inc.).

Results

Thirteen independent transformants were analyzed using FISH, fiber-FISH, and Southern hybridization to determine structurally how transgenes were integrated into the wheat genome. To determine the chromosomal location of the transgene, we co-hybridized two repetitive clones, pSc119 from *S. cereale* and pAs1 from *Ae. tauschii*. The pSc119 FISH patterns identify all B-genome chromosomes and chromosomes 4A, 2D, 3D and 5D of hexaploid wheat (Rayburn and Gill 1985). The clone pAs1 is D genome-specific and identifies all

Fig. 1 Schematic representation of the plasmids pAHG11 (7.3 kb) and pGL2ubi-tlp (7.5 kb). The numbers in parenthesis indicate distances in kilobasepairs
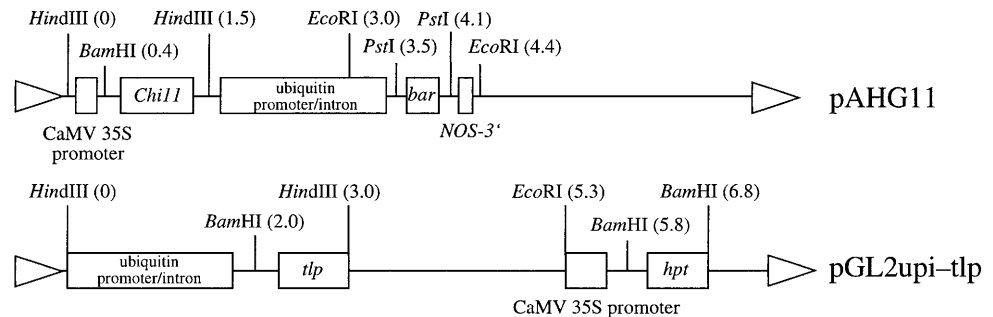
Fig. 2 C-banding and FISH patterns of the critical chromosomes in the transgenic lines and in the recipient wheat cultivar Bobwhite (BW). The transgene gene is visualized in yellow-green FITC fluorescence, and pSc119/pAs1 FISH sites are visualized in red rhodamine fluorescence
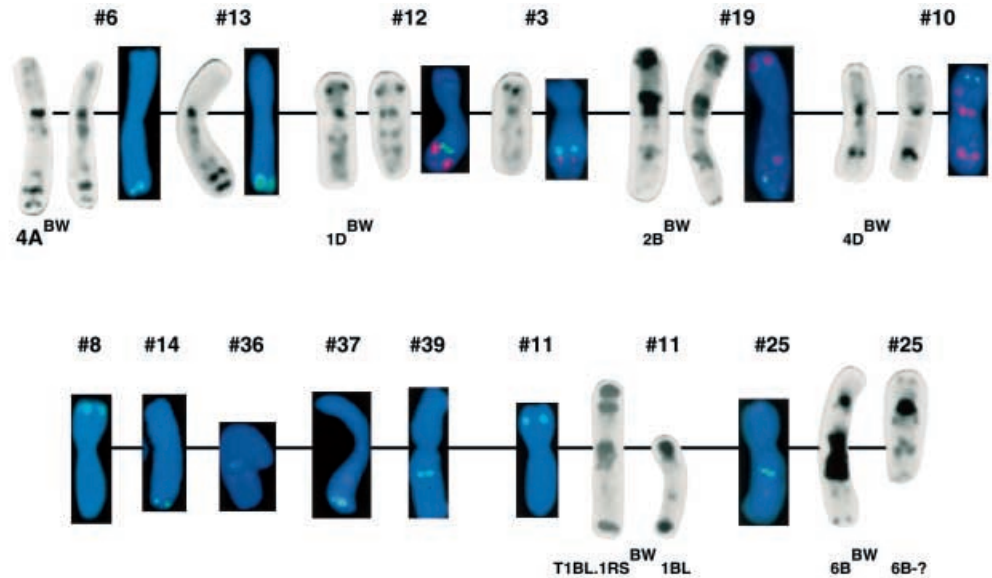

Table 1 Data from FISH experiments on extended DNA fibers (fiber-FISH) and metaphase chromosomes (FISH) using the plasmid pAHG11 as a probe. Size of the FISH signals was based on measurements of 3 to 5 fibers per line; –: size of the FISH signal was too small for reliable measurement

Type	Line	Fiber-FISH		Chromosome location
		μm	kb	
Type I	#6	27.0 ± 0.8	77.5	4AL, telomeric
Type II	#11	14.8 ± 1.9	42.5	?S, distal
	#13	43.4 ± 1.5	124.6	4AL, telomeric
	#14	11.2 ± 0.1	32.1	6AL, telomeric
	#19	25.9 ± 0.6	74.3	2BL, subtelomeric
Type III	#3	2.8 ± 0.4	8.0	1DL, interstitial
	#8	–	–	?S, telomeric
	#10	2.5 ± 0.1	7.2	4DS, telomeric
	#12	–	–	1DL, interstitial
	#25	–	–	?L, proximal
	#36	–	–	?L, interstitial
	#37	2.6 ± 0.2	7.5	?L, telomeric
	#39	–	–	?L, proximal

the D-genome chromosomes. Thus, by co-hybridizing a mixture of pSc119 and pAs1 to chromosome preparations, all B- and D-genome chromosomes and 4A of wheat can be identified. C-banding analysis was performed on all transgenic lines using the recipient wheat cultivar, Bobwhite, as a control for determining whether the integration of the transgene was associated with cytologically detectable chromosomal rearrangements. The chromosomal location of the trangene in lines #3, #6, #10, #12, #13, #14 and #19 was determined unambiguously (Table 1, Fig. 2). In none of these lines was the insertion of the transgene associated with chromosomal rearrangements detected by C-banding analysis.

With our fiber-FISH technique, 1 μm of microscopic distance is equivalent to 2.87 kb of DNA (Jackson et al. 1998). Therefore, distances measured digitally can be converted into kilobases of DNA and be used to deter-

mine the approximate copy number of the transgenes. Three basic patterns of integration were observed in these lines: Type I, a large insertion of tandemly repeated transgenes; Type II, an insertion of several copies of tandemly repeated plasmid DNA interspersed with unknown DNA over a large physical distance; and Type III, an insertion of one or two copies of the plasmid, which may or may not be disrupted by unknown DNA.

Type I-large tandem loci

FISH of metaphase chromosomes of line #6 mapped the transgene in the telomeric region of the long arm of chromosome 4A that is marked by a telomeric C-band (Figs. 2 and 3a, Table 1). Co-hybridization of the two repetitive DNA elements, pSc119 and pAs1, revealed a physical association of these repetitive elements with the transgene on metaphase chromosomes. Fiber-FISH analysis of this line showed a large continuous insertion (Fig. 3b). The size of this transgene insert was 77 kb (Table 1), equivalent to the length of approximately 11 copies of the transgene plasmid. The large gap in the signal (Fig. 3b, arrow) was consistent in all of the digital images collected, suggesting that there is a gap of about 7 kb in the transgene locus of this line. Based on the fiber-FISH data, the transgenic locus was not coincident with either of the two repetitive elements used in this study (Figs. 2 and 3b). This apparent discrepancy between the results from the two techniques is probably caused by the high condensation level of the DNA within a metaphase chromosome, whereas closely spaced loci can be separated on extended DNA fibers.

Type II-large tandem interspersed loci

A different integration pattern of the transgene was observed in lines #11, #13, #14 and #19. Metaphase FISH
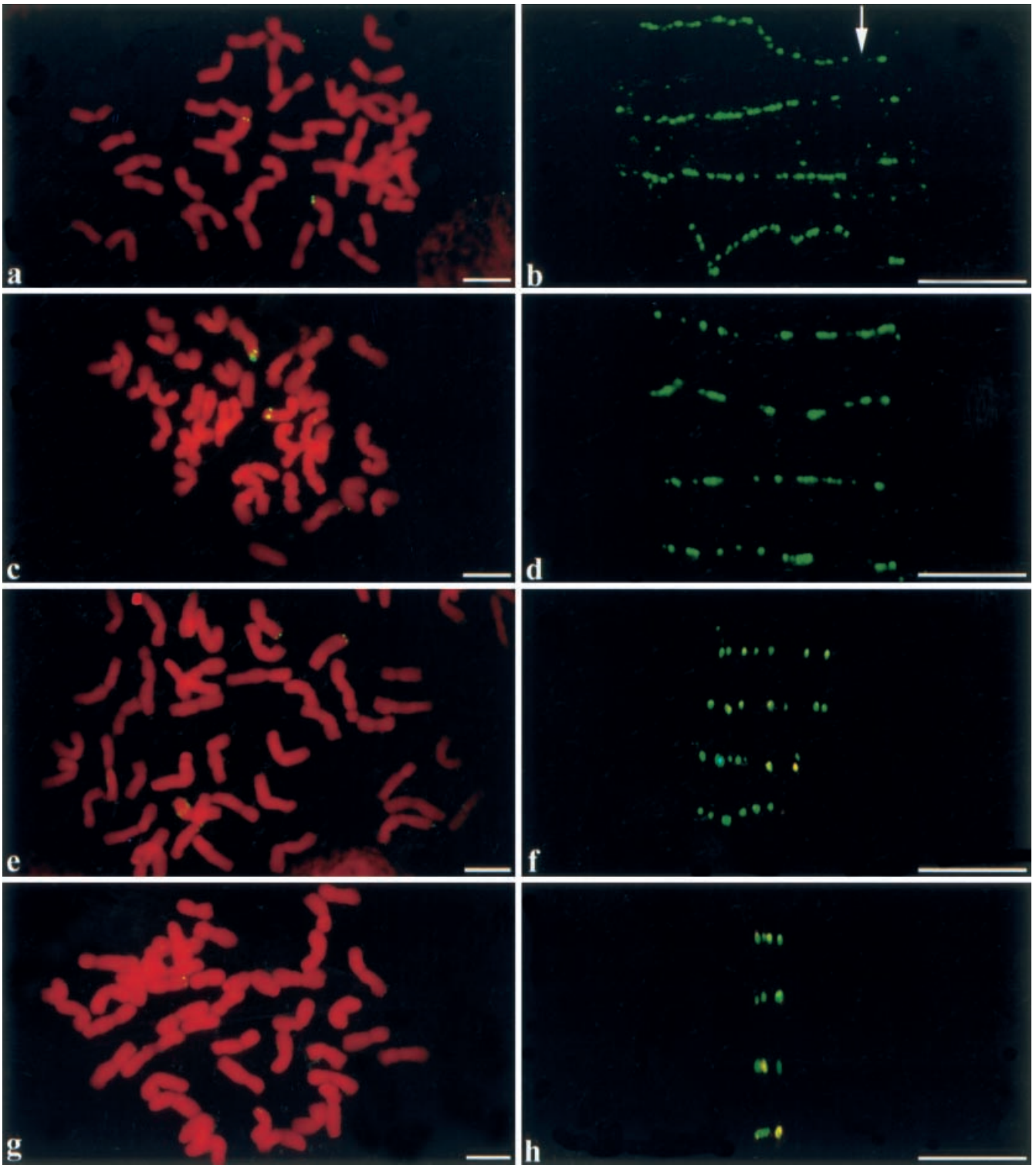
Fig. 3a–h FISH of transgenes on chromosomes (**a, c, e** and **g**) and extended DNA fibers (**b, d, f** and **h**) of transformed wheat lines. **a** and **b** Type I integration pattern: line #6, the 7.3-kb plasmid is inserted in the form of large tandem repeats of about 77 kb, representing about 11 copies of the transgene. **c** and **d** Type II integration pattern: line #19, the 7.3-kb transgene is inserted in the form of large tandem repeats that are interspersed with unknown DNA. **e** and **f** line #14, Type II integreation pattern spanning about 32 kb, which is equivalent to the length of four copies of either of the two plasmids pAHG11 and pGL2ubi-tlp used in transformation. **g** and **h** Type III integration pattern: line #10, small loci of about 7 kb equivalent to approximately one copy of the transgene. The transgene is visualized in yellow-green FITC. Bars are equal to 10 µm

mapped the transgene in line #19 in the telomeric C-band-positive region of the long arm of chromosome 2B (Figs. 2 and 3c). In line #13 the transgene was mapped in the telomeric C-band-positive region of chromosome arm 4AL. The chromosomal location of the transgene in line #11 could not be determined unambiguously because of the lack of diagnostic pSc119/pAs1 FISH sites (Fig. 2).

Fiber-FISH analyses of lines #11, #13, #14 and #19 revealed large physical loci with transgenes interspersed by unknown DNA (Fig. 3d, Table 1). These loci ranged from 42 kb up to 125 kb in size. The size of the individual continuous signal was larger than the basic 7.3-kb plasmid, which was used as a probe. Thus, the smaller loci hybridizing to the probe within the larger physical locus consist of multiple copies of the integrated whole (or nearly whole) plasmids.

Transgenic line #14 was the only line that was cotranformed with the plasmids pGL2ubi-tlp and pAHG11 (Chen et al. 1999). By using Chen et al. (1999) sequential N-banding, FISH, and segregation analyses, Chen et al. (1998, 1999) mapped both transgenes to the same location in the telomeric region of the long arm of chromosome 6A. In this line, the transgenes were inserted in the form of tandem repeats interspersed with unknown DNA. The size of the locus was about 32 kb, which may contain one or possibly two copies each of the two plasmids used in transformation (Fig. 3e and f, Table 1).

Type III-small, low-copy loci

The third integration pattern was observed in lines #3, #8, #10, #12, #25, #36, #37 and #39 where there were small loci representing a single copy, or at the most two copies, of the transgene (Fig. 3h, Table 1). It was impossible to tell from fiber-FISH analyses whether these transgenes were clustered or dispersed throughout the genome. However, only single loci were detected from FISH on metaphase chromosomes, suggesting a single chromosomal locus that may include several small transgene loci. Fiber-FISH is limited in that if physically connected sequences are separated by more than a few hundred kb of DNA it is difficult to tell if they are on the same DNA fiber. This is a result of slide preparation during which the DNA fibers do not line up linearly, but tend to cross adjacent fibers resulting in a network of DNA fibers.

Discussion

Agrobacterium-mediated transformation is a complex process whereby usually only one or a few copies of a transgene are integrated into the host genome with rearrangement of T-DNA and host DNA (reviewed in Tinland 1996). Multiple copies of a rearranged T-DNA were found in potato, *Solanum tuberosum* L., using the fiber-FISH method (Wolters et al. 1998). Integration of multiple copies of a transgene has also been reported

from biolistic methods. In hexaploid oat, *Avena sativa* L., Pawlowski and Somers (1998) observed the integration of several copies of a transgene at a single genetic locus spanning up to 280 kb interspersed by unknown DNA.

Thirteen transgenic wheat plants were analyzed using FISH and fiber-FISH to determine more accurately the physical structure of the transgene loci. These plants had been analyzed previously for the expression and segregation of transgene loci (Chen et al. 1998, 1999). Phenotypic and Southern blot data indicated that the transgenes segregated in a Mendelian fashion. The Southern blot data also indicated that different lines had variable copy numbers of the transgenes and represented independent transformation events (Chen et al. 1998).

FISH has been used previously to determine physically where transgenes are located on metaphase chromosomes (Hoopen et al. 1996). The transgenes, along with two genome-specific repetitive elements (pSc119 and pAs1) and C-banding analysis, were used in this study to elucidate the chromosomal location of the transgene and determine if they integrated into constitutive heterochromatic regions as indicated by the repetitive DNA probes. FISH data indicated that there was no preference for a specific genome, chromosome, or chromosome arm location. A few of the lines had transgenes integrated into C-heterochromatin as indicated by co-localization of pSc119 or pAs1 and the transgene on metaphase chromosomes. The fiber-FISH data indicated that the transgene did not integrate into highly repetitive pSc119/pAs1 sites in all the lines analyzed. However, because pSc119 and pAs1 are only two out of several repeat families, we cannot exclude the possibility that integration of the transgene occurred in or near another repeat family that comprise the C-heterochromatin.

Fiber-FISH is a variation on the FISH technique that uses extended DNA fibers as target molecules instead of condensed chromosomes (Heng et al. 1992; Parra and Windle 1993). The resolution of this technique is close to the Watson-Crick double helix (about 2.94 kb/μm). In our hands, the calibration is 2.87 kb/μm. This technique was used to more closely examine the physical structure of the transgene loci. The fiber-FISH analysis revealed that the pattern of integration was highly variable even though small (presumably single plasmid) insertions were most likely. Insertion of transgenic plasmid was found to occur with or without interspersed unknown DNA. There was clean-cut evidence for multiple copies of the transformed plasmid at the same locus in several lines, which is consistent with the segregation data of Chen et al. (1998, 1999).

The fiber-FISH data also clarify the Southern blot and copy number data reported previously for these 13 lines by Chen et al. (1998). Based on the number of different-sized bands detected by the transgene probe we had concluded that transgenic lines #11, #13, #19 (and several others) had multiple copies of the transgenes (*chi11* and *bar*). The fiber-FISH analysis has confirmed this conclusion and provided an estimated copy number ranging from 6 to 17. The fiber-FISH results also demonstrate

the difficulties of estimating copy numbers when tandem insertions of the transgenic plasmid result in one or two strong bands in Southern blots. Thus, we had expected line #6 to contain a small number of copies of the transgenic plasmid, but fiber-FISH analysis indicates that the transgenic locus may contain up to 11 copies of the transgenic plasmid. However, because the whole plasmid was used as a probe in FISH experiments, but only the transgene was used in Southern blot analysis, it is possible that the higher copy number indicated by FISH consists of plasmid sequences. In some cases the Southern blot analysis has resulted in larger estimates of the transgene copy number that resulted from plant DNA interspersions within the transgene(s). This is clearly the case for lines #10, #12 and #37, which had multiple bands in the Southern blot, whereas the fiber-FISH data suggest a single copy of the transgenic plasmid. It is possible that these lines have several copies of the transgene that are separated by more than a few hundred kb of plant DNA, and thus could not be mapped to the same DNA fiber. In other cases, the data from both methods are consistent. It is clear that fiber-FISH gives a much clearer picture of the clustering and length of the transgenic locus than Southern blotting.

The integration of transgenes from biolistic transformation techniques is poorly understood. Transgenes are hypothesized to integrate at sites of replication fork clusters (Pawlowski and Somers 1998) or through a two-step process where transgenes form co-integrates in the plant cell before being integrated at a 'hot spot' (Kohli et al. 1998). Our current data support both of these models. Multimerized copies were observed in Type I patterns, and the interspersed mode of integration in Types II and III indicate that there may be hot spots for integration. Whether these sites are regions of clustered replication forks is not clear.

In hexaploid oat, the integration of the transgene in lines produced by biolistic bombardment was associated with chromosomal rearrangements (Svitashev et al. 2000). However, no structural rearrangements associated with the integration of the transgene were observed in the present study. In six lines (#6 & #8, 4AL; #3 & #12, 1DL; #9, 2BL; #10, 4DS), the chromosomal location of the transgene was determined unambiguously by the pSc119/pAs1 FISH patterns, or as in the case of line #14 (6AL) by sequential N-banding and FISH analyses. C-banding analysis revealed no evidence for chromosomal rearrangements around the integration sites in these lines. Furthermore, C-banding did not detect any differences in banding patterns compared with those of the recipient wheat cultivar Bobwhite in the remaining transgenic lines, except for lines #11 and #25. Line #11 segregates for the wheat-rye translocation chromosome T1BL.1RS from Bobwhite and a telocentric chromosome identified as the long arm of chromosome 1B. Line #25 segregates for a normal chromosome 6B and a T6B-? translocation chromosome (Fig. 2). In both lines, these rearrangements were not associated with the integration of the transgene and are more likely induced by the tissue culture conditions (Sacristan 1971; Orton 1980; Larkin and Scowcroft 1981; Lapitan et al. 1984). Thus, chromosomal rearrangements are not necessarily associated with the integration of transgenes in lines produced by biolistic bombardment.

The implications of understanding the mode of transgene integration and its effect on transgene expression are important. Previous studies reported transgene silencing from multiple copies of the transgene and integration into regions of C-heterochromatin (Dobie et al. 1996; Dorer and Henikoff 1997). Very few of the transgenic lines expressed the *chi11* gene, which was under the control of the CaMV 35S promotor beyond the T_0 generation, whereas all lines expressed the *bar* gene that was driven by the maize ubiquitin promotor. Line #14 was co-transformed with the plasmids pAHG11 and pGL2ubi-tlp, both of which integrated at the same locus in the telomeric region of the long arm of chromosome 6A (Chen et al. 1999). In this plant, only the *bar* and the *tlp* genes driven by the ubiquitin promotor were expressed, whereas neither *chi11* nor *hpt* under the control of the CaMV 35S promotor were expressed.

The structural analysis of transgene loci presented here has clarified some of these issues. In the transgenic plants analyzed, the type of promoter used was most critical for the expression of the transgene in wheat. Only the *bar* and *tlp* genes driven by the ubiquitin promoter were expressed in all the lines, whereas neither *chi11* nor *hpt* under the control of the CaMV 35S promoter was expressed in any of the lines. Contrary to other reports, there was no silencing of the transgenes that were associated with C-heterochromatin (Dobie et al. 1996; Dorer and Henikoff 1997; Fanti et al. 1998).

Furthermore, neither copy number, integration type, nor the location of the integration site in relation to proximal, interstitial, and distal eu- and hetero chromatic regions affected the expression of the genes under the control of the ubiquitin promoter.

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